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Pro-inflammatory cytokines predominate in the brains of dogs with visceral leishmaniasis: A natural model of neuroinflammation during systemic parasitic infection

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ABSTRACT

Visceral leishmaniasis is a multisystemic zoonotic disease that can manifest with several symptoms, including neurological disorders. To investigate the pathogenesis of brain alterations occurring during visceral leishmaniasis infection, the expression of the cytokines IL-1β, IL-6, IL-10, IL-12p40, IFN-γ, TGF-β and TNF-α and their correlations with peripheral parasite load were evaluated in the brains of dogs naturally infected with *Leishmania infantum*. IL-1β, IFN-γ and TNF-α were noticeably up-regulated, and IL-10, TGF-β and IL-12p40 were down-regulated in the brains of infected dogs. Expression levels did not correlate with parasite load suggestive that the brain alterations are due to the host's immune response regardless of the phase of the disease. These data indicate the presence of a pro-inflammatory status in the nervous milieu of dogs with visceral leishmaniasis especially because IL-1β and TNF-α are considered key factors for the initiation, maintenance and persistence of inflammation.

1. Introduction

Leishmaniasis is a complex disease caused by more than 20 different species of protozoans of the *Leishmania* genus (Kinetoplastida, Trypanosomatidae). Leishmaniasis is transmitted by over 30 distinct phlebotomine sand flies and has four different types of clinical presentation: visceral leishmaniasis (VL), cutaneous leishmaniasis, muco-cutaneous leishmaniasis, and post-kala-azar dermal leishmaniasis (PKDL). Leishmaniasis is an important cause of human death, among the parasitic diseases it is surpassed only by malaria (Chappuis et al., 2007). VL, also known as kala-azar, is an anthropozoonosis caused by the *Leishmania donovani* complex: *L. infantum* (Syn. *chagasi*) in the Americas and in the Mediterranean basin, and *L. donovani* in Asia and Africa (Baneth et al., 2008; Lukeš et al., 2007; Mauricio et al., 2000).

In addition to the skin, VL affects mainly the organs with cells of the mononuclear phagocyte system, such as the spleen, lymph nodes, liver, bone marrow. The typical histopathological finding is granulomatous inflammatory reaction associated with the presence of *Leishmania* amastigotes within macrophages (Alvar et al., 2004; Baneth et al., 2008). Although there are several reports of the systemic symptoms of VL, such as fever, anemia, weight loss, skin disease, and renal and ocular alterations (Alvar et al., 2004; Blavier et al., 2001; Ciaramella and Corona, 2003; Moreno et al., 1998), few studies have related the occurrence of injuries in the central nervous system (CNS).

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In humans, the involvement of the peripheral nervous system in VL is more usual than the involvement of the CNS (Snydman et al., 2006). Hashim et al. (1995) reported that 46% of patients with VL presented neurological symptoms, most of them describing the sensation of burning feet. In addition to peripheral neuropathy, there are reports of alterations similar to those in Guillain–Barré syndrome, cranial nerve dysfunction and meningitis (Snydman et al., 2006). Dogs with VL may present signs of generalized CNS involvement, with seizures, cranial nerve alterations, vestibular and cerebellar signs, motor incoordination, tetraparesis and tetraplegia, and myoclonia (Font et al., 2004; Ikeda et al., 2007; José-López et al., 2012; Melo et al., 2012). The main histopathological alterations in the brains of these dogs are meningitis and choroiditis, which are mainly secondary to antigen and immunoglobulin deposition and leukocyte infiltration (Garcia-Alonso et al., 1996; Ikeda et al., 2007; Melo et al., 2009; Melo and Machado, 2009, 2011; Nieto et al., 1996; Viñuelas et al., 2001).

VL is an immunomediated disease, and studies have demonstrated a failure of the cellular response in symptomatic dogs that is characterized by a diminishment of the lymphoproliferative response against *Leishmania* antigens and a decrease in the number of $CD4^+$ T lymphocytes (Pinelli et al., 1999; Solano-Gallego et al., 2000). In infected but clinically healthy dogs, the Th1 immune response prevails and is mediated by IL-2, IFN-γ and TNF-α. This is in contrast to symptomatic animals, in which cytokines such as IL-4, IL-10 and TGF-β mediate the Th2 response (Barbiéri, 2006; Reis et al., 2009; Solano-Gallego et al., 2011; Strauss-Ayali et al., 2005). Nevertheless, immune response polarization is not a rule during *Leishmania* infection due to the role of regulatory T cells (Treg), which suppress the immune response by means of cell-cell interactions and/or production of suppressor cytokines, such as IL-10 and TGF-β, which render Th0 the immune response against *Leishmania* (Belkaid et al., 2002; Campanelli et al., 2006; Gantt et al., 2003).

In view of the paucity of available data relating VL to the CNS and because the presence of cytokines in the nervous milieu may indicate an inflammatory environment, the aims of this study were to determine the gene expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-12p40, IFN-γ and TNF-α and the anti-inflammatory cytokines IL-10 and TGF-β in the brains of dogs naturally infected with VL and to determine the correlation between the brain cytokine profile and the peripheral parasite load.

2. Materials and methods

2.1. Animals

Twenty-five dogs, 13 male and 12 female, ranging in age from 1 to 6 years old that were referred to the Veterinary Teaching Hospital of UNESP, São Paulo State University in Araçatuba, São Paulo State, Brazil were included in this study. Nineteen naturally infected dogs, which were euthanized with the owners' permissions, in compliance with state law (São Paulo, 2006), as soon as the VL diagnosis was confirmed were included in the infected group, and six uninfected healthy dogs, with no underlying condition and whose deaths had no sign of nervous involvement (e.g. trauma) were included in the control group. VL diagnosis was achieved using a routine ELISA (enzyme-linked immunosorbent assay) according to Lima et al. (2005). None of these animals were previously vaccinated against CVL. The animals were also negative for toxoplasmosis and neosporosis, as assessed by indirect immunofluorescence assays.

2.2. Sampling

Blood samples were collected, and the dogs were euthanized with an overdose of pentobarbital (Nembutal®) and potassium chloride. Necropsies were performed immediately after euthanasia, and macroscopic lesions were recorded. Brains were collected and separated into two hemispheres; one of which was placed in 10% buffered-formalin, and fragments of 0.5 cm^3 from the thalamus, hippocampus, piriform/temporal cortex and periventricular white matter were collected from the other hemisphere and stored in RNAlater (Applied Biosystems, AM7020) and frozen at −80 °C. Fragments of 2 cm^3 of spleen were also collected and immediately frozen at −80 °C.

2.3. Brain cytokine quantification by RT-qPCR

Total RNA was extracted from the brain samples stored in RNAlater weighing ca. 100 mg using the RiboPure kit (Applied Biosystems, AM1924) according to the manufacturer's protocol. After RNA extraction, any potential contaminating DNA was removed by incubation with DNAse I (Applied Biosystems, AM1928) at 37 °C for 30 min. Next, RNA was quantified with a NanoDrop spectrophotometer (260/280 ratio between 2.0 and 2.3) and frozen at −80 °C until the reverse transcription reaction was preformed. cDNA production was achieved using the RETROscript kit (Applied Biosystems, AM1710) with 1000 ng of RNA and oligo(dT) primers in a final volume of 20 μL and cycled at 44 °C for 1 h and 92 °C for 10 min. Then, the cDNA was frozen at −20 °C until further analyses. Specific canine primers and hydrolysis probes for amplifying cytokines were selected based on the scientific literature (IL-1β, IL-6, IL-10, IL-12p40, IFN-γ and TGF-β) or designed (TNF-α) using the on-line software Primer3Plus[1] (Untergasser et al., 2007). The hydrolysis probes were labeled with the reporter dye FAM at the 5′ end, and with the quencher dye BHQ-1 at the 3′ end (Table 1). qPCR was carried out in a real-time thermocycler (Bio-Rad, CFX96) using TaqMan Master Mix (Applied Biosystems, 4304437), 400 nM of each primer, 250 nM of the probe and 5 μL of cDNA in a total volume of 25 μL. The amplification conditions were as follows: 55 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. For each cytokine, values of reaction efficiency, determination coefficients (r^2) and angular coefficients (slopes) were obtained from amplification of seven serial dilutions of a pool of cDNA. Quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method

[1] http://www.primer3plus.com.